ENGINEERING *ZYMOMONAS MOBILIS* FOR EFFICIENT FUEL ETHANOL PRODUCTION FROM LIGNOCELLULOSIC FEEDSTOCKS

BY

KORI LYNN DUNN

THESIS

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Adviser:

Associate Professor Christopher V. Rao

Abstract

The bacterium *Zymomonas mobilis* is of interest for the commercial production of fuel ethanol from lignocellulosic feedstocks due to its high ethanol productivity. *Z. mobilis* has been engineered to ferment all of the sugars present in cellulosic biomass, but the digestion of five carbon sugars by the strain is still slow and inefficient. The inefficiencies are present because *Z. mobilis* displays preferential sugar digestion—only after all six carbon sugars in the culture medium are depleted will five carbon sugars be consumed. This is not due to an internal regulatory mechanism, but is instead due to the fact that *Z. mobilis* cannot efficiently transport pentose sugars into the cell. Therefore, in this work, we investigated the effect on ethanol production of introducing a dedicated transporter for the five carbon sugar xylose in recombinant *Z. mobilis*. Specifically, the *xylE* transporter gene from *Escherichia coli* was expressed in a xylose-fermenting strain of *Z. mobilis* and glucose-xylose co-fermentation and xylose fermentation were analyzed. The engineered strain showed faster xylose consumption in the presence of glucose when the concentration of both sugars was high—in media containing 5% glucose and 5% xylose, 100% more xylose was consumed and 20% more ethanol was produced after 55 hours compared to the control strain. The engineered strain was also able to produce more ethanol in media containing a high concentration of xylose as sole carbon source, with over 100% more ethanol being produced after a 110-hour period. Additionally, the transporter also allowed for quantifiable ethanol production in media containing a very low percentage of carbon sources. These data indicate that introducing pentose-specific transporters in recombinant *Z. mobilis* is an effective approach toward engineering cellulosic ethanol production by the strain.

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Chapter 1: Introduction

1.1 Motivation

The United States spends over $200,000 per minute, or $13 million per hour, on foreign oil imports [1]. Due to growing demand and decreasing domestic production, our dependence on foreign fuel has only been increasing. This threatens our national security and leaves us vulnerable to oil-supply disruptions. It intensifies our political and economic vulnerability and is the largest contributor to our trade deficit. And because America has less than 3% of known oil reserves, we cannot simply drill our way out of the problem [2]. With demand outpacing supply, it has become clear that the United States needs to find alternative renewable energy sources that can be produced right here at home. With the enactment of the Renewable Fuel Standard (RFS) under the 2007 Energy Independence and Security Act, the United States mandated that 36 billion gallons of renewable fuels be blended into our transportation fuels every year by the year 2022 [3]. Only 15 billion gallons of this can be first-generation corn ethanol, and at least 16 billion gallons must be second-generation cellulosic fuels, or fuels produced from agricultural or municipal waste products. Therefore, by law and to protect our environment, lignocellulosic biofuel production technologies must be developed, optimized, and commercialized.

1.2 Cellulosic ethanol production by microorganisms

Of the cellulosic fuels under consideration for meeting the demands of the RFS, ethanol has emerged as a frontrunner due to its reduced greenhouse gas emissions compared to petroleum, its ability to be blended with gasoline for use in existing engines, and its ability to encourage rural economic growth [4]. Ethanol is produced by microorganisms that release it as a waste product in the metabolic process of converting sugars into energy. Several microorganisms have been

considered for fuel ethanol production from cellulosic biomass. Among the most popular are the bacteria *Escherichia coli* and *Zymomonas mobilis* and the yeast *Saccharomyces cerevisiae*. While *E. coli* is able to ferment a wide range of substrates into ethanol, it is limited by its low ethanol tolerance and productivity, its narrow pH range for growth, and the fact that it is publicly perceived to be dangerous [5]. *S. cerevisiae* is safe and can produce large amounts of ethanol, but it is limited by its susceptibility to contamination [6] and the production of by-products [7]. Although it possesses a narrow substrate range, *Z. mobilis* has emerged as the strongest candidate for cellulosic ethanol production. *Z. mobilis* has a higher specific ethanol production rate than any other organism under review [8]. It can resist contamination, and is even free from any known viral invaders. It displays a unique homoethanologenic metabolism that is not coupled to growth, which gives it a higher sugar uptake rate than any other ethanologenic organism. It also has an extremely high ethanol tolerance that is equal to if not better than that of yeasts [9].

1.3 Sugar metabolism in *Z. mobilis*

Z. mobilis ferments glucose, sucrose, and fructose to ethanol via the Entner-Doudoroff pathway together with the enzymes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (Adh) [10]. For every mole of sugar, 2 moles of ethanol, 2 moles of carbon dioxide, and 1 mole of energy, or ATP, is produced. *Z. mobilis* is unique in that it displays what is known as the "uncoupled growth phenomenon"—sugars are brought into the cell and fermented to ethanol regardless of growth requirements. This leads to the strain's very high ethanol productivity and also leads to a high specific rate of ATP synthesis. This extra ATP must be exhausted through an ATP spilling reaction, most likely carried out by the membrane F_0F_1-type H^+-ATPase in a manner that is similar to the reverse of the ATP synthesis reaction [11]. *Z. mobilis* is also unique

in that it uses a facilitated diffusion system for sugar transport. The single sugar transporter, the glucose facilitator (GLF) protein, requires no metabolic energy and has a very high affinity for glucose. In addition to the Entner-Doudoroff pathway, *Z. mobilis* contains most enzymes of the Embden-Meyerhof-Parnass (EMP) pathway, and many in the tricarboxylic acid (TCA) cycle, but lacks most enzymes of the pentose phosphate pathway (PPP) [12].

1.4 Optimizing *Z. mobilis* for fuel ethanol production

Hydrolysis of lignocellulosic biomass yields a mixture primarily composed of the six carbon sugar glucose and the five carbon sugars xylose and arabinose [13]. Because it lacks the PPP, *Z. mobilis* is not inherently able to digest the five carbon sugars present in this biomass. However, researchers have conferred the ability to ferment both xylose [14] and arabinose [15] on the strain. For xylose fermentation this required the expression in *Z. mobilis* of two xylose assimilation genes, xylose isomerase (*xylA*) and xylulokinase (*xylB*), as well as two enzymes from the non-oxidative branch of the PPP, transketolase (*tktA*) and transaldolase (*talB*), all from *E. coli*. Similarly, for arabinose fermentation, *tktA* and *talB* were expressed together with the *araBAD* operon, the arabinose assimilation genes from *E. coli* (Figure 1). While capable of growth on pentose sugars as sole carbon source, these strains still showed sequential and inefficient pentose digestion in the presence of glucose. Sequential sugar digestion is undesirable; simultaneous sugar digestion is an important characteristic of a successful large-scale ethanologen because of the continuous nature of industrial fermentations—glucose will always be present in the cellulosic culture medium, the pentose sugars will never be digested, and yields will be reduced [16]. While sequential sugar digestion in many bacterial species is attributable to carbon catabolite repression, a regulatory mechanism acting at the genetic level,

there is no inherent genetic mechanism present in *Z. mobilis*. Instead, due to its highly specific GLF transporter, pentose sugars simply are unable to enter the cell when glucose is present [15].

1.5 Approach of presented research

Giving *Z. mobilis* a dedicated transporter for the pentose sugar xylose should improve the efficiency of digestion of this sugar both in the presence and absence of glucose, ultimately leading to more efficient cellulosic ethanol production by the strain (Figure 2). The gene encoding a pentose sugar transporter, like those for pentose metabolism, can be taken from *E. coli*, which has a dedicated xylose transporter known as XylE [17]. This *E. coli* transporter makes use of proton motive force (PMF) to function—since *Z. mobilis* has an abundance of PMF that is normally dissipated by the F_0F_1-type H^+-ATPase, it should not present an additional metabolic burden for the cells. Therefore, this transporter was cloned under the control of the promoter of *Z. mobilis*'s native sugar transporter, GLF, to ensure appropriate expression levels of the protein. The effect of this sugar transporter on sugar consumption in *Z. mobilis* was subsequently analyzed.

1.6 Figures

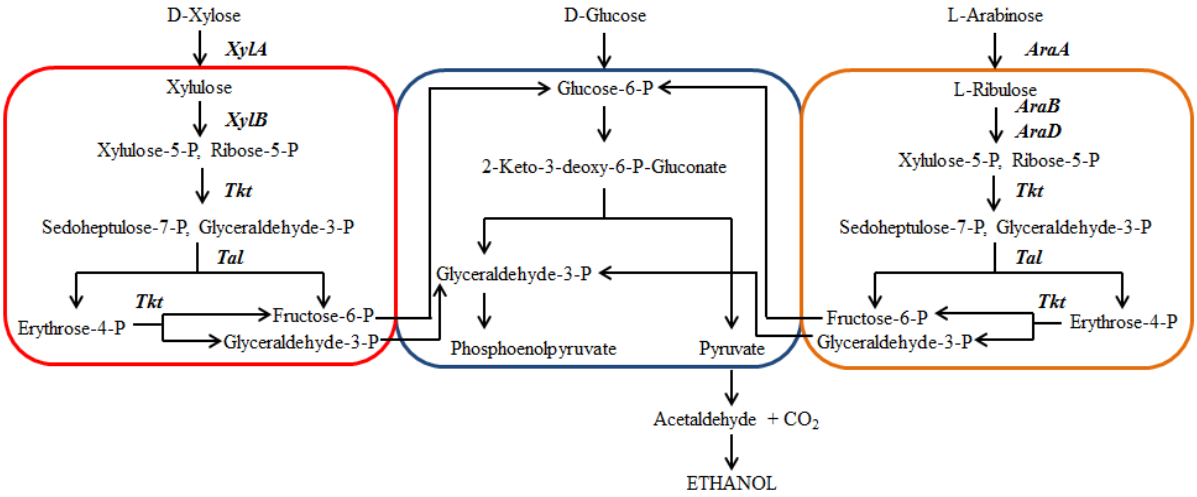

Figure 1: Simplified mechanism of xylose and arabinose assimilation in engineered *Z. mobilis*. Both sugars ultimately enter the Entner-Doudoroff pathway, shown in the center, leading to ethanol formation. Abbreviations: XylA, xylose isomerase; XylB, xylulokinase; Tkt, transketolase; Tal, transaldolase; AraA, L-arabinose isomerase; AraB, L-ribulokinase; AraD, L-ribulose-5-phosphate-4-epimerase.

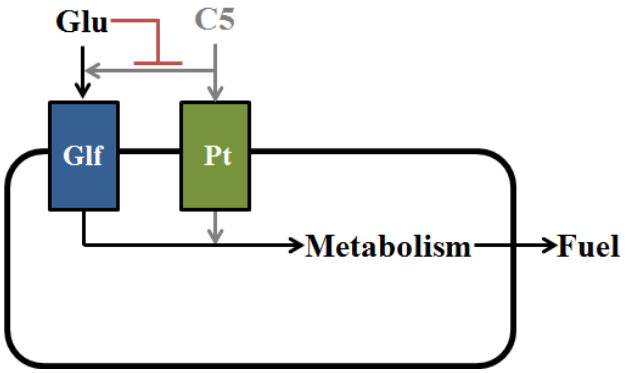

Figure 2: Working model for sugar utilization in *Z. mobilis*. A single transporter, GLF, mediates the uptake of glucose (Glu) and the pentose sugars (C5). However, when glucose is present in the growth medium, the pentose sugars are unable to be transported into the cell. By introducing a pentose-specific transporter (Pt), we can enable multiple sugar utilization and increase the rate of sugar fermentation in *Z. mobilis* and, as a result, improve ethanol production.

Chapter 2: Materials and Methods

2.1 Bacterial strains, media, and culture conditions

E. coli DH5α [F^- Φ80d*lacZ*ΔM15 Δ(*lacZYAargF*) U169 *deoR recA1 endA1 hsdR17* (rK^- mK^+) *supE44* λ^- *thi-1 gyrA96 relA1*] was used as the cloning host. *E. coli* MG1655 [F^- λ^- *ilvG- rfb-50 rph-1*] was used as the source of all *E. coli* genes and was the parent of all chromosomal knockouts. *E. coli* strains GM2929 [F^- *ara-14 leuB6 thi-1 fhuA31 lacY1 tsx-78 galK2 galT22 glnV44 hisG4 rpsL136 xyl-5 mtl-1 dam13::Tn9 dcm-6 mcrB1 hsdR2* (rK^- mK^+) *mcrArecF143*] and GM119 [F^- *dam-3 dcm-6 metB1 lacY1 galK2 galT22 tonA31 tsx-78 supE44 mtl-1 (thi-1)*] were obtained from the *E. coli* Genetic Stock Center of Yale University and were used as sources of unmethylated plasmid DNA. *Z. mobilis* ATCC 31821 (ZM4) was used as the transformation host. *Z. mobilis* ATCC 10988 was used as the source of *Z. mobilis* native plasmid DNA. *E. coli* strains were cultured in LB medium (Difco) or M9 minimal medium supplemented with 0.2% glucose or xylose, as necessary, and containing chloramphenicol (20 µg/ml), kanamycin (40 µg/ml), ampicillin (100 µg/ml), or spectinomycin (100 µg/ml) as appropriate. *E. coli* cultures were grown at 37°C, except when harboring temperature-sensitive plasmids, when cultures were grown at 30°C. *Z. mobilis* strains were grown in rich medium (RM) containing yeast extract (10 g/liter), potassium phosphate, dibasic (2 g/liter), glucose or xylose at the designated concentration, and chloramphenicol (100 µg/ml) or spectinomycin (100 µg/ml) as appropriate. All *Z. mobilis* cultures were grown at 30°C.

2.2 Construction of plasmids encoding xylose-metabolism genes

Few recombinant *Z. mobilis* strains have emerged and genetic manipulation of the strain has proven difficult. Transformation efficiency is low and plasmid maintenance is an issue [18]. As

such, 3 different plasmids with 3 different origins of replication were constructed (Table 1) and each was subsequently tested for transformation ability and stability in *Z. mobilis*.

The first *Z. mobilis* replicative plasmid that was constructed contained open reading frame 2 (*ORF2*) from the *Z. mobilis* native plasmid pZMO3 (pZM2). *ORF2* was deemed necessary for plasmid replication in *Z. mobilis* [19]. The 887 bp fragment carrying *ORF2* was amplified from pZMO3 isolated from *Z. mobilis* ATCC 10988 using primers KD054F (ATGCGAATTCCGGTTTTAATTTTACAAATACCC) and KD054R (ATGCGGTACCACTGAATTTATTCTGATTCGTCTTG) and inserted into the vector pPROTet.E (Clontech Laboratories) containing a chloramphenicol resistance gene and the ColE1 origin of replication for maintenance in *E. coli*. The *xylAB* operon from its start codon and including its native terminator sequence was amplified from *E. coli* MG1655 genomic DNA using primers KD047F (GTTAGGAGATAAACATGCAAGCCTATTTTGACCAGCTCGATCGCG) and KD047R (ATGCCTCGAGGCCATTAACAAATGATTTTCAGAATA). The 310bp strong and constitutive glyceraldehyde-3-phosphate promoter (P_{gap}) was amplified from *Z. mobilis* ZM4 genomic DNA using primers KD046F (ATGCGAATTCACTTTGTTCGATCAACAACCCG) and KD046R (GTTTATTCTCCTAACTTATTAAGTAGCTACTATATTCC). The P_{gap} and *xylAB* gene fragments were then fused using the method of gene splicing by overlap extension (SOE) [20] and the constructed synthetic operon was then inserted into pPROTet.E containing *ORF2*. The *talB* gene was amplified from start to stop codon from *E. coli* MG1655 genomic DNA using primers KD050F (AAGAAAGGTTTCGATATGACGGACAAATTGACCTC) and KD050R (ATGCGGGCCCTTACAGCAGATCGCCGATCA). The 308bp strong and constitutive enolase promoter (P_{eno}) was amplified from *Z. mobilis* ZM4 genomic DNA using

primers KD049F (ATGCGGTACCCCTTCATGTTTTGCTTCATG) and KD049R (ATCGAAACCTTTCTTAAAATCTTTTAGAC). The P_{eno} and *talB* gene fragments were then fused using the SOE approach and subsequently inserted into pPROTet.E containing *ORF2* and P_{gap}-*xylAB*. The *tktA* gene containing its native ribosome binding site sequence and its native terminator sequence was amplified from *E. coli* MG1655 genomic DNA using primers KD051F (ATGCGGGCCCTCATCCGATCTGGAGTCAAAATGTCCTCACG) and KD051R (ATGCGCGGCCGCCCGCAAACGGACATATCAAGG) and cloned into pPROTet.E containing *ORF2*, P_{gap}-*xylAB*, and P_{eno}-*talB* directly downstream of the *talB* stop codon to produce the P_{eno}-*talB*-*tktA* synthetic operon and completing construction of plasmid pKD7.

The second *Z. mobilis* replicative plasmid contained the entire *Z. mobilis* native plasmid pZMO3 as maintenance region. The 2.7kb pZMO3 fragment was amplified from pZMO3 isolated from *Z. mobilis* ATCC 10988 using primers KD086F (ATGCGAATTCCCCGAGGAATATCATTTAACAA) and KD086R (ATGCGGTACCAATTTAAAAACTCCCTAATTTTCG) and inserted into the plasmid pPROTet.E. The synthetic operons P_{gap}-*xylAB* and P_{eno}-*talB*-*tktA* were constructed as before and inserted into pPROTet.E containing pZMO3 to yield plasmid pKD8.

The third *Z. mobilis* replicative plasmid was constructed in plasmid pJS71, which contains the broad-host-range origin from plasmid pBBR1 and was kindly provided by Dr. Jeffrey Skerker. pBBR1 was isolated from *Bordetella bronchiseptica* S87 and has been shown to replicate in a wide range of Gram-negative bacteria [21]. The P_{gap}-*xylAB* synthetic operon was amplified from plasmid pKD8 using primers KD103F (ATGCCTCGAGACTTTGTTCGATCAACAACCCG) and KD103R (ATGCTCTAGAGCCATTAACAAATGATTTTCAGAATA) and inserted into pJS71. The P_{eno}-*talB*-*tktA* synthetic operon was amplified from plasmid pKD8 using primers

KD101F (ATGCTCTAGACCTTCATGTTTTGCTTCATG) and KD101R (ATGCGAGCTCCCGCAAACGGACATATCAAGG) and inserted into pJS71 containing P_{gap}-*xylAB* to yield plasmid pKD9.

2.3 Confirmation of functionality of the cloned xylose metabolism genes in *E. coli*

Complementation analysis was performed in *E. coli* knockout mutants unable to grow on xylose as sole carbon source to confirm functionality of the genes cloned into all 3 *Z. mobilis* plasmids. *E. coli* MG1655 knockouts were constructed using the standard method of Datsenko and Wanner [22]. A *xylA*-deficient mutant was created in *E. coli* MG1655 carrying the Red recombinase genes on plasmid pKD46 with a chloramphenicol-resistance cassette amplified from plasmid pKD3 using primers KD003F (ACTGAAAGGGAGTGCCCAATATTACGACATCATCCATCACGTGTAGGCTGGAGCTG CTTC) and KD003R (CGGTATCGCTACCGATAACCGGGCCAACGGACTGCACAGTCATATGAATATCCTCC TTAG). The resistance gene was subsequently removed, leaving behind only an FLP recognition target (FRT)-site scar using plasmid pCP20 [23]. A *xylB* knockout was previously constructed by Dr. Tasha Desai using the same technique that was used for construction of the *xylA* mutant. Testing the functionality of the transaldolase B gene cloned into the *Z. mobilis* plasmids required the construction of a mutant deficient in function of both isozymes of the transaldolase protein, *talA* and *talB,* as well as deficient in the function of the phosphofructokinase (*pfkA*) enzyme. PfkA, together with the enzyme fructose-1,6-bisphosphate aldolase (FbaA), is able to bypass the function of the transaldolase enzymes in the pentose phosphate pathway in converting sedoheptulose-7-phosphate into erythrose-4-phosphate, so transaldolase mutants are still able to

grow on xylose as sole carbon source when *pfkA* is present [24]. This triple mutant was constructed via the method of Datsenko and Wanner using primers KD078F (ATGAACGAGTTAGACGGCATCAAACAGTTCACCACTGTCGGTGTAGGCTGGAGCTG CTTC), KD078R (TTATAGTTTGGCGGCAAGAAGATCTTCCAGTTTGCGTTGACATATGAATATCCTCCT TAG), KD079F (ATGACGGACAAATTGACCTCCCTTCGTCAGTACACCACCGGTGTAGGCTGGAGCTG CTTC), KD079R (TTACAGCAGATCGCCGATCATTTTTTCCAGTTTTTCCTGGCATATGAATATCCTCCTT AG), KD097F (ATGATTAAGAAAATCGGTGTGTTGACAAGCGGCGGTGATGGTGTAGGCTGGAGCTG CTTC) and KD097R (TTAATACAGTTTTTTCGCGCAGTCCAGCCAGTCACCTTTGCATATGAATATCCTCCTT AG). Testing the functionality of the transketolase A gene cloned into the *Z. mobilis* plasmids required the construction of a mutant deficient in the function of both isozymes of the transketolase protein, *tktA* and *tktB*. This double mutant was created using primers KD080F (ATGTCCTCACGTAAAGAGCTTGCCAATGCTATTCGTGCGCGTGTAGGCTGGAGCTG CTTC), KD080R (TTACAGCAGTTCTTTTGCTTTCGCAACAACGTTATCAACACATATGAATATCCTCCT TAG), KD081F (ATGTCCCGAAAAGACCTTGCCAATGCGATTCGCGCACTCAGTGTAGGCTGGAGCTG CTTC) and KD081R (TCAGGCACCTTTCACTCCCAGCACCTTATGCGCTTTTGCCCATATGAATATCCTCCTT

AG). Plasmids were introduced into *E. coli* knockout strains using electroporation, and strains were then analyzed for their ability to grow on xylose as sole carbon source. All cloned genes were deemed functional by way of these complementation analyses. An example of the resulting data can be seen in Figure 3.

2.4 Transformation of plasmid DNA encoding xylose-metabolism genes into *Z. mobilis*

As stated previously, the efficiency of transformation of plasmid DNA into *Z. mobilis* is extremely low and plasmid stability is an issue. Therefore, 3 plasmids with 3 different maintenance regions were tested for transformation efficiency and stability in *Z. mobilis*. Variables in the electroporation protocol were systematically altered until the optimal transformation conditions were determined. Both methylated plasmid DNA, isolated from *E. coli* DH5α, and unmethylated plasmid DNA, isolated from *E. coli* GM119 or GM2929, were tested, either at a low concentration of approximately 200ng per 40µL of competent cells or at a higher concentration of approximately 1µg per 40µL of competent cells. A variety of competent-cell preparation procedures were tested. Cells were harvested during exponential phase, late exponential phase, or stationary phase, and subsequently treated with lysozyme at a concentration of 0 or 1 ng/µL. Cells were washed with deionized water and 10% glycerol and resuspended in 10% glycerol for transformation. The TypeOne restriction inhibitor (Epicentre Technologies) was included in the transformation mixture at a concentration of 0.5µL, 0.75µL, or 1µL per 40µL of competent *Z. mobilis* cells. Transformation was performed using a Bio-Rad gene pulser XCell total system set at 0.8, 1.6, 1.8, 2.3, 2.8, or 3.0kV, 200Ω and 25mF. Cuvettes with 0.1cm gaps were used. Cells were allowed to grow at 30°C for 1, 3, or 6 hours following transformation and before plating on selective media. Plates were allowed to incubate at 30°C for

2-5 days until colonies appeared. The first successful transformant was isolated from a competent cell preparation harvested at late exponential phase, not treated with lysozyme, containing 0.5µL of TypeOne per 40µL of cells and a high concentration of unmethylated plasmid DNA. The gene pulser was set at 1.6kV and the outgrowth time was 1 hour. Of the 3 plasmid constructs, this first transformant contained the pBBR1-based plasmid pKD9. Presence of the plasmid was confirmed by plasmid isolation from the *Z. mobilis* cells using the GeneJET plasmid miniprep kit (Thermo Scientific) and subsequent transformation into *E. coli* DH5α. The isolate with plasmid presence confirmed was used for subsequent analysis.

2.5 Isolation of a *Z. mobilis* transformant capable of growth in media containing xylose as sole carbon source

While the *Z. mobilis* cells were confirmed to have plasmid pKD9, they still were unable to grow on xylose as sole carbon source. It has been previously reported that *Z. mobilis* cells require a period of adaptation to the sugar [25]. As such, cells were grown first in RM containing glucose as sole carbon source, and subsequently transferred into media containing 4% glucose and 1% xylose, 2.5% glucose and 2.5% xylose, 1% glucose and 4% xylose, and finally 5% xylose. The first isolate capable of growth on xylose as sole carbon source, designated KD67, was used for subsequent analysis.

2.6 Construction of a plasmid encoding a xylose-specific transporter

The 310bp glucose facilitator protein promoter (P_{glf}) was amplified from *Z. mobilis* ZM4 chromosomal DNA using primers KD125F (ATGCTCTAGATTTTTTAAAAAGAAAACTGTTTTTTTAAACACTTATGTTGC) and

KD125R (GGCGATTCCTCTCCCGCC). The *xylE* gene was amplified from its start codon and including its native terminator sequence using primers KD126F (GGCGGGAGAGGAATCGCCATGAATACCCAGTATAATTCCAGTTATATATTTTC) and KD126R (ATGCTCTAGAGAATTTATCGCGCTGATTGTGAAGTA). The P_{glf} and *xylE* gene fragments were then fused using the method of gene splicing by overlap extension (SOE) [20] and the constructed synthetic operon was inserted into the plasmid pEZCm, kindly provided by Dr. Jeffrey Skerker, to form plasmid pKD10.

2.7 Confirmation of functionality of the cloned xylose transporter in *E. coli* and transformation into *Z. mobilis*

Complementation analysis was performed in a transport-deficient mutant of *E. coli* to confirm functionality of the cloned *xylE* gene. Plasmid pKD10 was inserted by electroporation into an *E. coli* strain previously constructed by Dr. Tasha Desai that lacks the XylE, AraE, XylFGH, and AraFGH transporters and is therefore not able to grow on media containing xylose as sole carbon source. Because pKD10 restored this ability, the cloned *xylE* gene was deemed functional. Plasmid pKD10 was then introduced into *Z. mobilis* using the previously optimized electroporation protocol. Presence of the plasmid was confirmed by plasmid isolation from the *Z. mobilis* cells using the GeneJET plasmid miniprep kit (Thermo Scientific) and subsequent transformation into *E. coli* DH5α. The isolate with plasmid presence confirmed, designated KD633, was used for subsequent analysis.

2.8 Fermentation and evaluation of strain performance

Recombinant *Z. mobilis* strains were cultivated in RM in sealed 15mL polypropylene tubes filled to 60% volume with shaking at 30°C until stationary phase, at which time they were used as seed culture for fermentations. Fermentations were performed in RM in sealed 50mL polypropylene tubes filled to 90% volume with shaking at 30°C. During fermentation, cell growth was monitored by the optical density at 600nm (OD_{600}). The concentration of glucose, xylose, and ethanol was monitored by filtering samples taken during fermentation through a 0.22µm polyethersulfone syringe filter and analyzing the resulting liquid using high performance liquid chromatography (HPLC). A Shimadzu HPLC system with a refractive index detector (Shimadzu RID-10A) was used together with an Aminex HPX-87H carbohydrate analysis column and a cation H micro-guard cartridge (Bio-Rad) kept at a temperature of 65°C. The mobile phase was 5mM H_2SO_4, pumped at a flow rate of 0.6mL/min. Peaks were identified and quantified by retention time comparison to authentic standards (glucose, xylose, and ethanol). All data are the average of biological triplicates.

2.9 Tables and figures

Plasmid	Relevant characteristics	Source or reference
pEZCm	Cm^R, pACYC184-pZMO1	Dr. Jeffrey Skerker
pJS71	Sp^R, pBBR1MCS-*lacZα*	Dr. Jeffrey Skerker
pPROTet.E	Cm^R, oriColE1	Clontech Laboratories
pZMO3	*Z. mobilis* native plasmid	[19]
pCP20	Amp^R, Cm^R, pSC101, *FLP*+, λ *c*I857+, λ p_R Rep^{ts}	[23]
pKD3	Amp^R, FRT-Cm^R-FRT, oriR6Kgamma	[22]
pKD46	Amp^R, pSC101-P_{BAD}-*γ*-*β*-*exo*, Rep^{ts}	[22]
pKD7	pPROTet.E-*ORF2*-P_{gap}*xylAB*-P_{eno}*talB*-*tktA*	This study
pKD8	pPROTet.E-pZMO3-P_{gap}*xylAB*-P_{eno}*talB*-*tktA*	This study
pKD9	pJS71-P_{gap}*xylAB*-P_{eno}*talB*-*tktA*	This study
pKD10	pEZCm-P_{glf}-*xylE*	This study

Table 1: Plasmids used in this study.

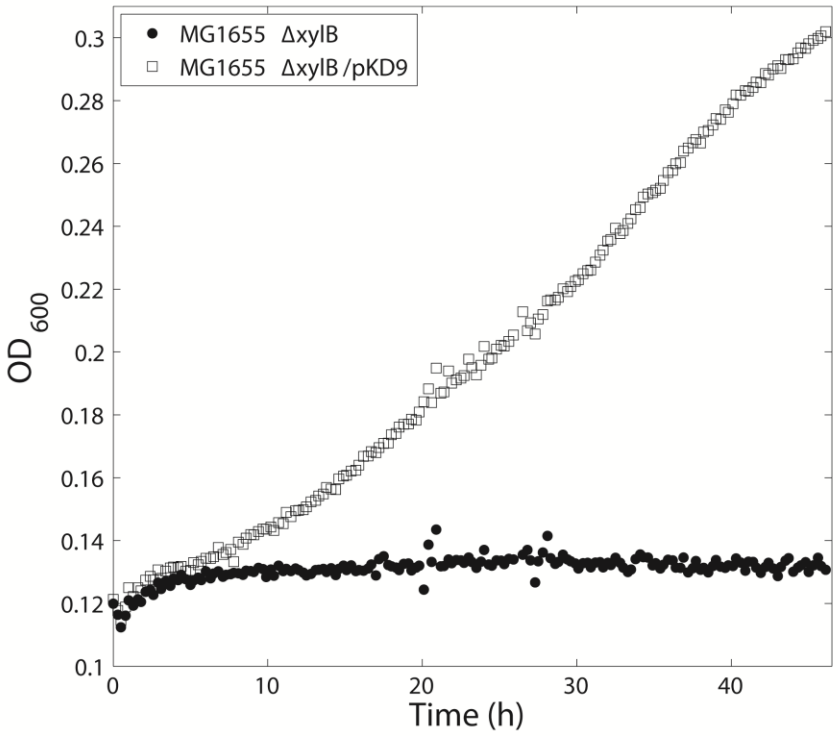

Figure 3: Complementation analysis of the cloned xylulokinase gene in plasmid pKD9. The *xylB* gene encoding the xylulokinase xylose-assimilation enzyme present in plasmid pKD9 is deemed functional due to its ability to restore growth on xylose as sole carbon source in an *E. coli* strain MG1655 *xylB* mutant.

Chapter 3: Results and Discussion

3.1 Isolation of a xylose-fermenting strain of *Z. mobilis* expressing a xylose-specific transporter

To develop a *Z. mobilis* strain capable of fermenting xylose, expression plasmids were constructed to introduce four *E. coli* xylose metabolism genes (xylose isomerase, xylulokinase, transaldolase, and transketolase) into *Z. mobilis*. Functionality of the cloned genes was confirmed by complementation analyses in *E. coli* deficient mutants. After electroporation of the plasmid into *Z. mobilis*, the first isolate to emerge from an adaptation process that was capable of growth on xylose as sole carbon source was designated KD67. Xylose digestion by this strain was slow and inefficient. Therefore, to improve xylose fermentation by KD67, an expression plasmid containing the xylose-specific transporter *xylE* gene from *E. coli* under the control of the *Z. mobilis GLF* promoter was developed. Functionality of the cloned transporter gene was confirmed by complementation analysis in an *E. coli* deficient mutant. The plasmid was then electroporated into KD67 to yield the xylose-fermenting transporter-expressing strain of *Z. mobilis* designated KD633.

3.2 Fermentation performance of KD633 in glucose-xylose co-culture

Several experiments were performed to evaluate the fermentation performance of KD633 in glucose-xylose mixed culture. Strains KD633, KD67, and the wild-type *Z. mobilis* were grown in rich media containing 5% glucose and 5% xylose (5%G-5%X), 2.5% glucose and 2.5% xylose (2.5%G-2.5%X), 1% glucose and 1% xylose (1%G-1%X) or 0.1% glucose and 0.1% xylose (0.1%G-0.1%X) as carbon sources. Each strain was analyzed for its rate of glucose and xylose consumption and its rate of ethanol production. Although true simultaneous sugar digestion was

not seen under any of the conditions analyzed, the strain containing the xylose-specific transporter did show better fermentation performance overall. In 5%G-5%X media (Figure 4), all three strains consumed glucose at near identical rates, showing that expression of the xylose metabolism genes and expression of the XylE transporter does not have an effect on glucose metabolism. However, strain KD633 was able to consume xylose at a faster rate than KD67. After 55 hours, KD633 had consumed approximately twice as much xylose as strain KD67, while the wild-type strain had consumed none at all. Further, the transporter-expressing strain also produced approximately 20% more ethanol than the transporter-lacking strain after 55 hours. Performance of strain KD633 was comparable to strain KD67 at the intermediate sugar concentrations, 2.5%G-2.5%X and 1%G-1%X (Figures 5 and 6), but improvement was again seen at the lowest sugar concentration, 0.1%G-0.1%X (Figure 7). Although the strain without the transporter reached a higher OD_{600}, the transporter enabled a maximum ethanol concentration that was over 200% higher than was seen in the xylose-fermenting strain lacking the transporter. These fermentation data demonstrate that strain KD633 outperforms strain KD67 and the wild-type *Z. mobilis* in media with very high and very low glucose and xylose concentrations.

3.3 Fermentation performance of KD633 in xylose culture

To further illustrate the superiority of strain KD633, several experiments were performed to evaluate its fermentation performance in xylose culture. Strains KD633, KD67, and the wild-type *Z. mobilis* were grown in rich media containing 10% xylose (10%X), 5% xylose (5%X), 1% xylose (1%X) or 0.1% xylose (0.1%X) as carbon source. Each strain was analyzed for its rate of xylose consumption and its rate of ethanol production. In media containing the largest amount of xylose, 10%X (Figure 8), the strain with the transporter reached a higher OD_{600}, consumed about

28% more xylose, and produced over 100% more ethanol after 110 hours. Again fermentation performance by KD633 and KD67 was comparable at the intermediate sugar concentrations, 5%X and 1%X (Figures 9 and 10, although KD633 did produce slightly more ethanol), but improvement was again seen at the lowest xylose concentration, 0.1%X (Figure 11). The transporter enabled a quantifiable maximum ethanol production, about 0.8g/L, while the strain without the transporter was unable to produce ethanol at this sugar concentration. These fermentation data demonstrate that strain KD633 outperforms strain KD67 and the wild-type *Z. mobilis* in media with very high and very low xylose concentrations.

3.4 Discussion

In order for lignocellulosic ethanol to be a competitive alternative fuel, the production process must be cost-effective. A cost-effective production process requires a microorganism capable of fermenting all sugars present in the cellulosic feedstock. While *Zymomonas mobilis* is a promising ethanologen, it is not able to efficiently ferment all of these sugars and displays preferential sugar digestion—no other sugars are consumed until glucose is exhausted from the culture medium. This is not due to an internal genetic regulatory mechanism, but is instead likely attributable to the fact that *Z. mobilis* has only one sugar transporter with a strong preference for glucose. Therefore, in this work, we constructed a strain of *Z. mobilis* capable of growth on xylose as sole carbon source by introducing four xylose metabolism genes from *E. coli*. Next, we introduced a xylose-specific transporter, XylE, also from *E. coli,* to enable xylose to enter the cell when glucose is present and to also improve ethanol production in media containing xylose as sole carbon source. The XylE transporter is a low-affinity high-capacity transporter, so it dominates when the sugar concentration outside the cell is high. Our results show that the

transporter has a positive effect on ethanol production in media containing 10% total carbon source and 0.1-0.2% total carbon source, but it has little effect on strain performance in media containing the intermediate sugar concentrations of 5, 2, and 1% total carbon source. We hypothesize that the positive results seen at the highest sugar concentrations are due to the optimal activity of the XylE transporter under these conditions. At the lowest sugar concentrations, we hypothesize that the cells without the transporter are in a starved state—thus causing them to divert all resources toward growth and to oxidize any ethanol that may be produced. On the other hand, the transporter enables an intracellular xylose concentration that is high enough to allow for temporary ethanol build-up in the culture medium at the expense of growth. As a whole, these data indicate that the expression of pentose-specific transporters in recombinant *Z. mobilis* is an effective approach toward improving cellulosic ethanol production by the strain. Future work toward this goal should include testing other xylose transporters, such as XylFGH from *E. coli* or the CC0814 xylose transporter from *Caulobacter crescentus*, as well as varying the expression levels of all xylose transporters by fusing them to different *Z. mobilis* native promoters. Additionally, a similar transporter analysis should be performed on an arabinose-fermenting strain of *Z. mobilis* using arabinose-specific transporters such as AraE or AraFGH from *E. coli*.

3.5 Figures

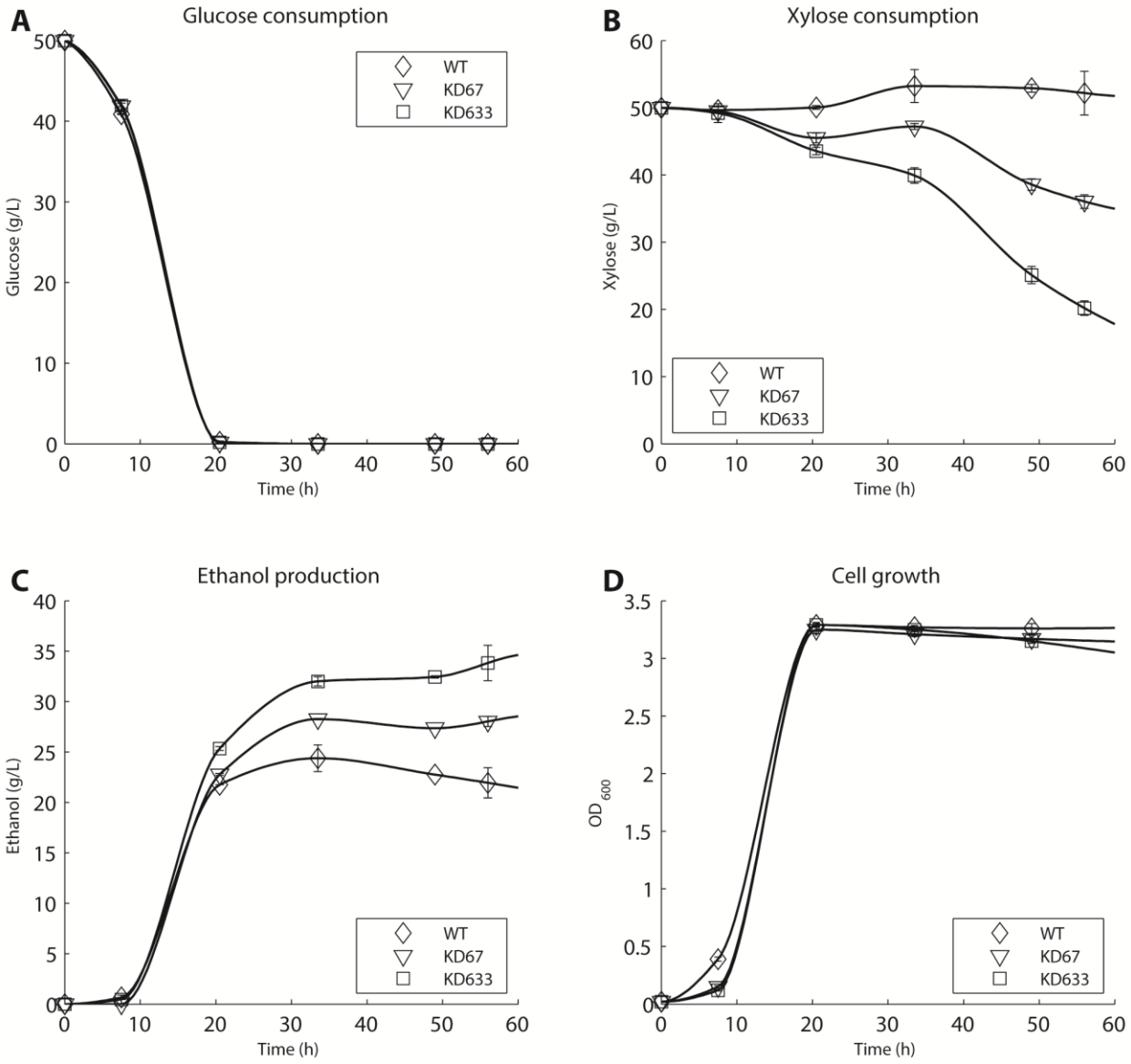

Figure 4: Strain analysis in rich medium containing 5% glucose and 5% xylose. Sugar concentrations, ethanol concentration, and growth are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A-C** Glucose, xylose, and ethanol concentrations for each strain **D** Growth as determined by the OD_{600}

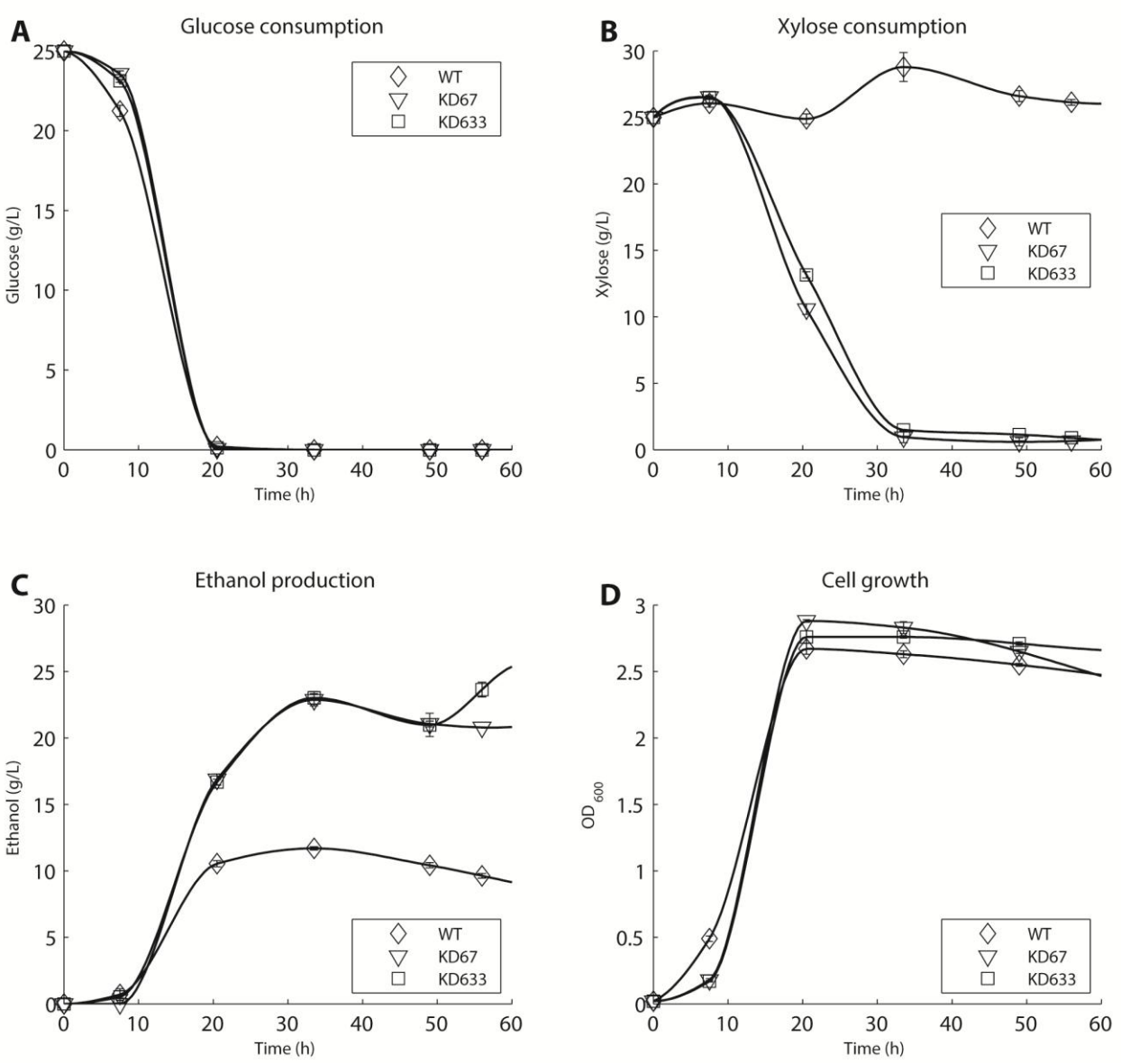

Figure 5: Strain analysis in rich medium containing 2.5% glucose and 2.5% xylose. Sugar concentrations, ethanol concentration, and growth are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A-C** Glucose, xylose, and ethanol concentrations for each strain **D** Growth as determined by the OD_{600}

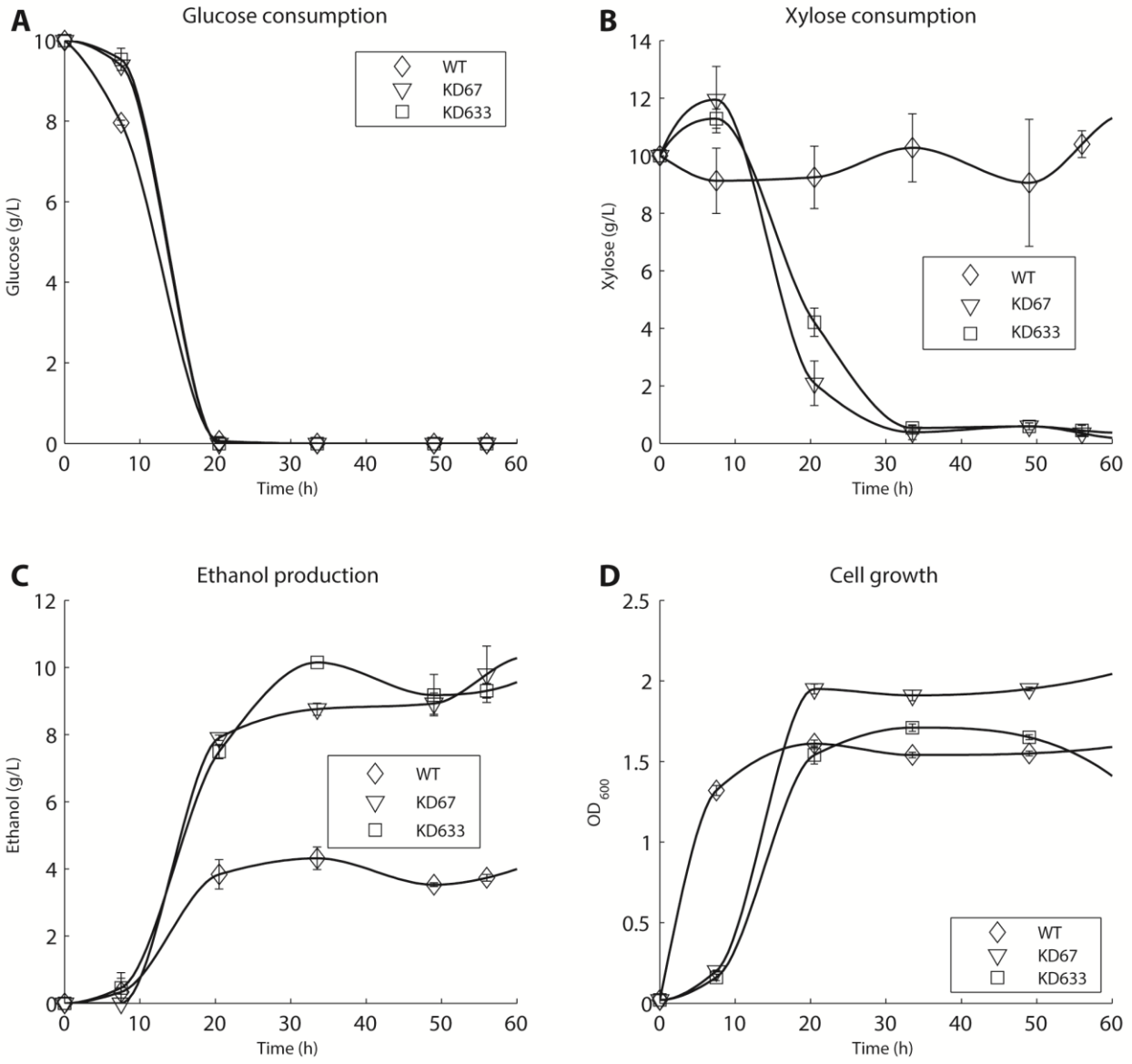

Figure 6: Strain analysis in rich medium containing 1% glucose and 1% xylose. Sugar concentrations, ethanol concentration, and growth are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A-C** Glucose, xylose, and ethanol concentrations for each strain **D** Growth as determined by the OD_{600}

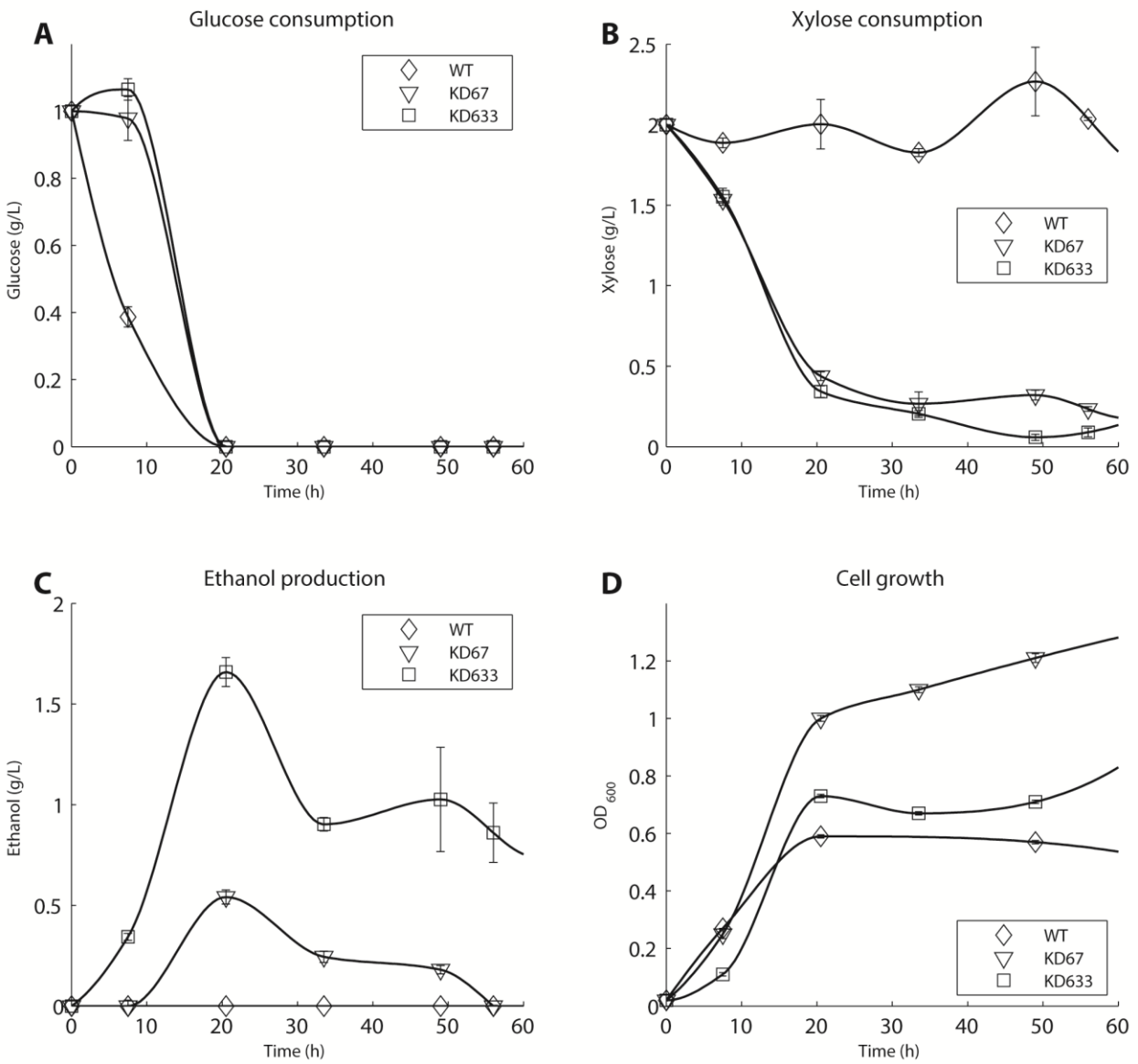

Figure 7: Strain analysis in rich medium containing approximately 0.1% glucose and 0.1% xylose. Sugar concentrations, ethanol concentration, and growth are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A**-**C** Glucose, xylose, and ethanol concentrations for each strain **D** Growth as determined by the OD_{600}

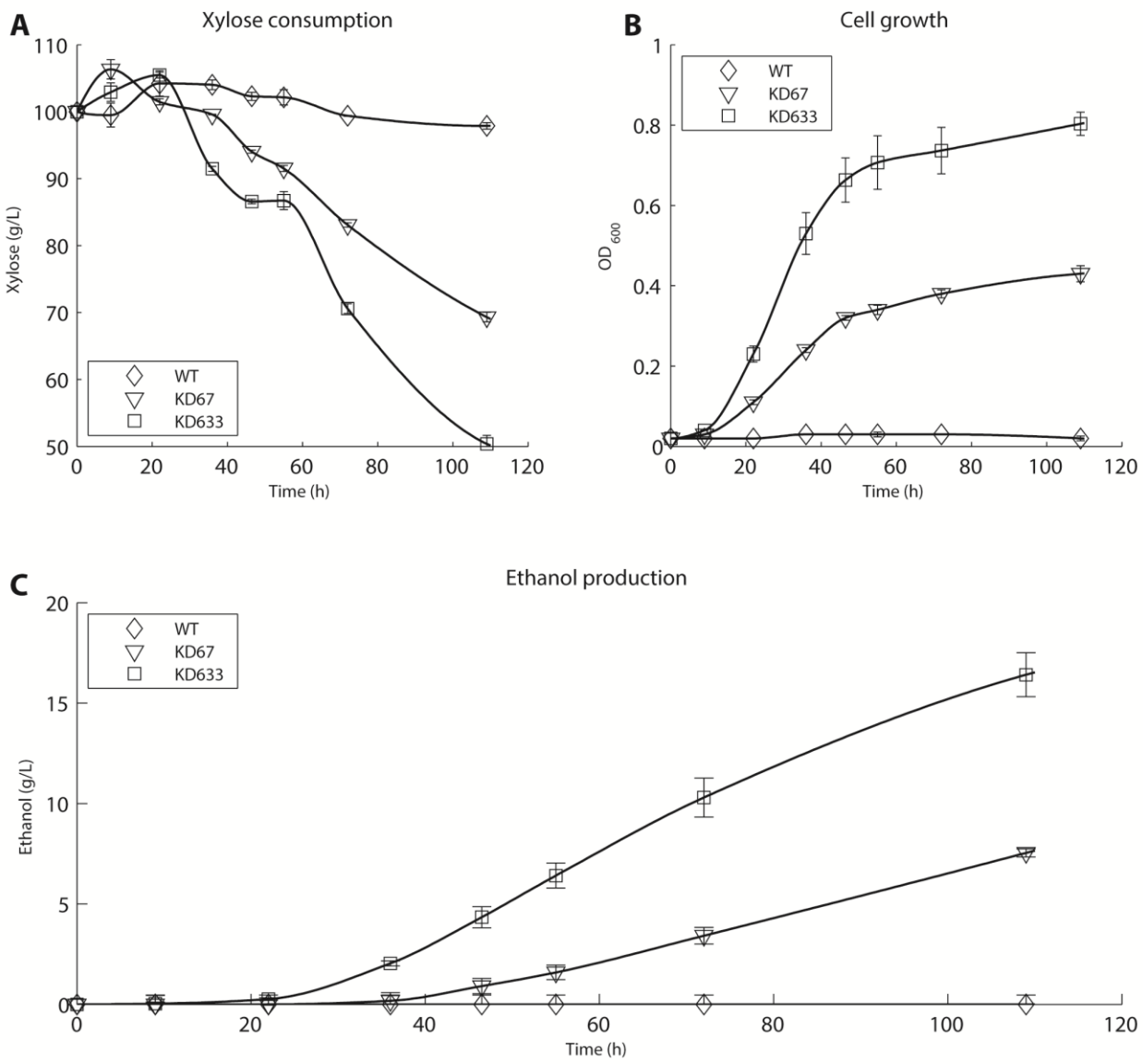

Figure 8: Strain analysis in rich medium containing 10% xylose. Xylose concentration, growth, and ethanol concentration are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A** Xylose concentration for each strain **B** Growth as determined by the OD_{600} **C** Ethanol concentration for each strain

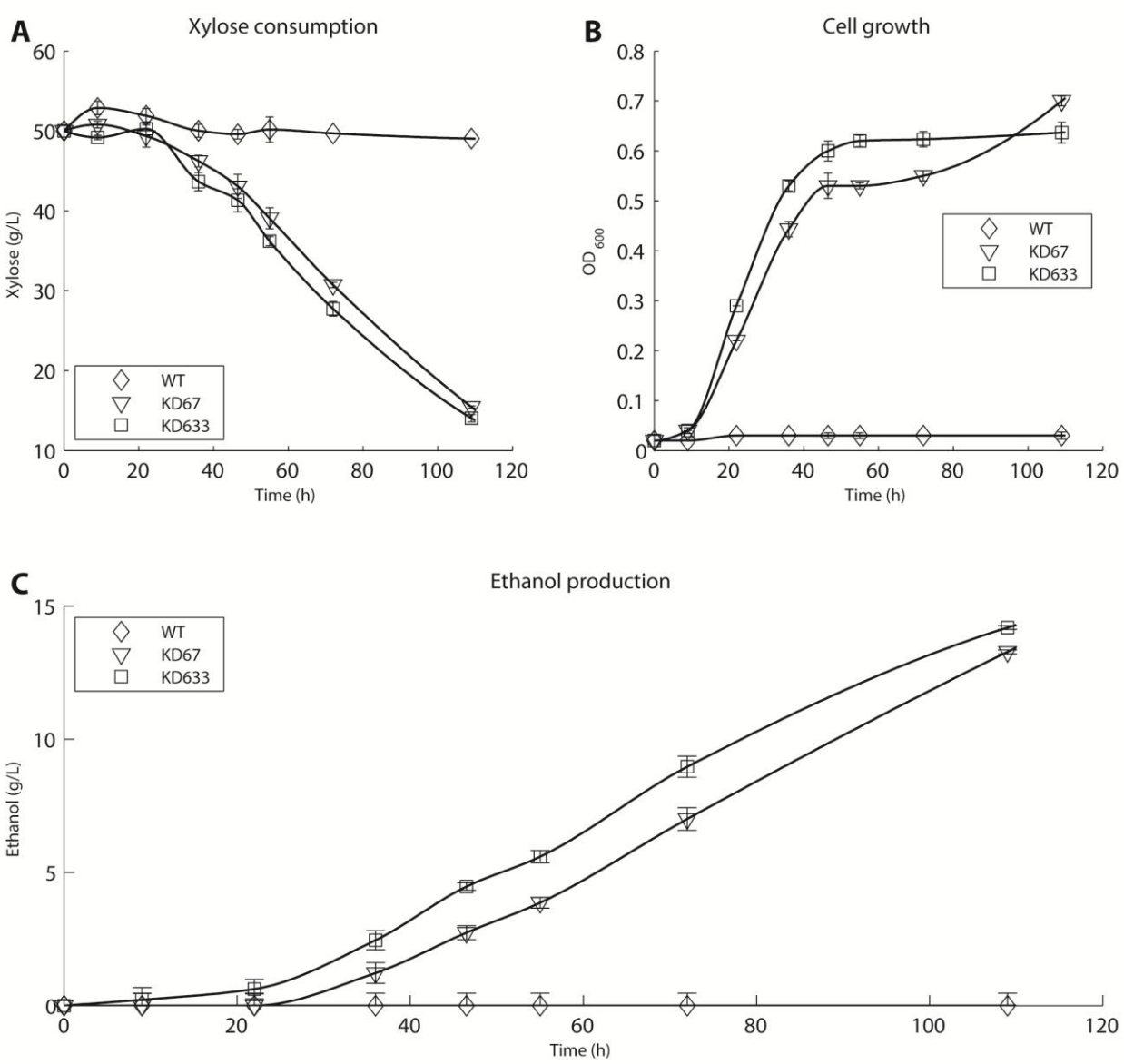

Figure 9: Strain analysis in rich medium containing 5% xylose. Xylose concentration, growth, and ethanol concentration are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A** Xylose concentration for each strain **B** Growth as determined by the OD_{600} **C** Ethanol concentration for each strain

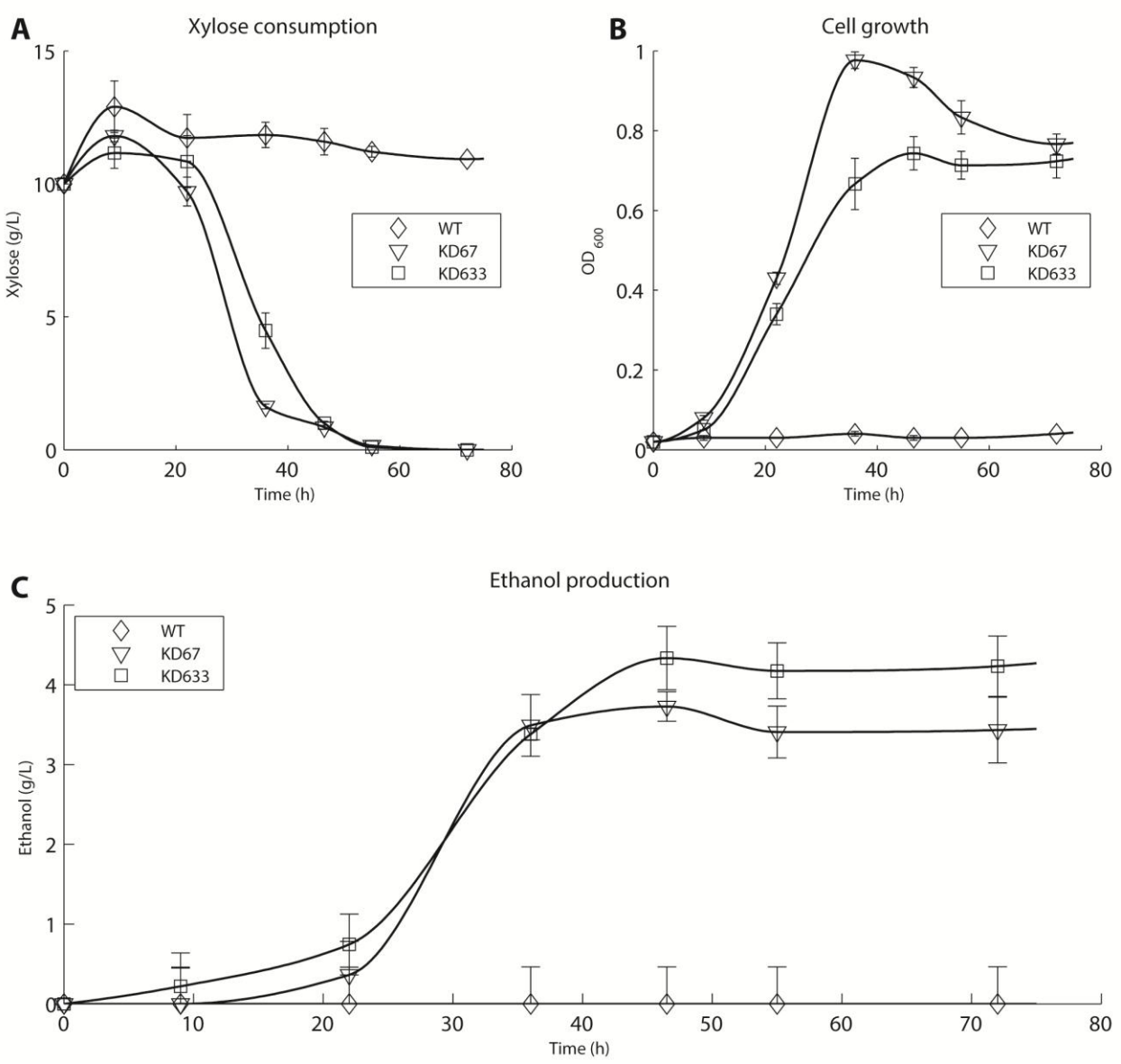

Figure 10: Strain analysis in rich medium containing 1% xylose. Xylose concentration, growth, and ethanol concentration are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A** Xylose concentration for each strain **B** Growth as determined by the OD_{600} **C** Ethanol concentration for each strain

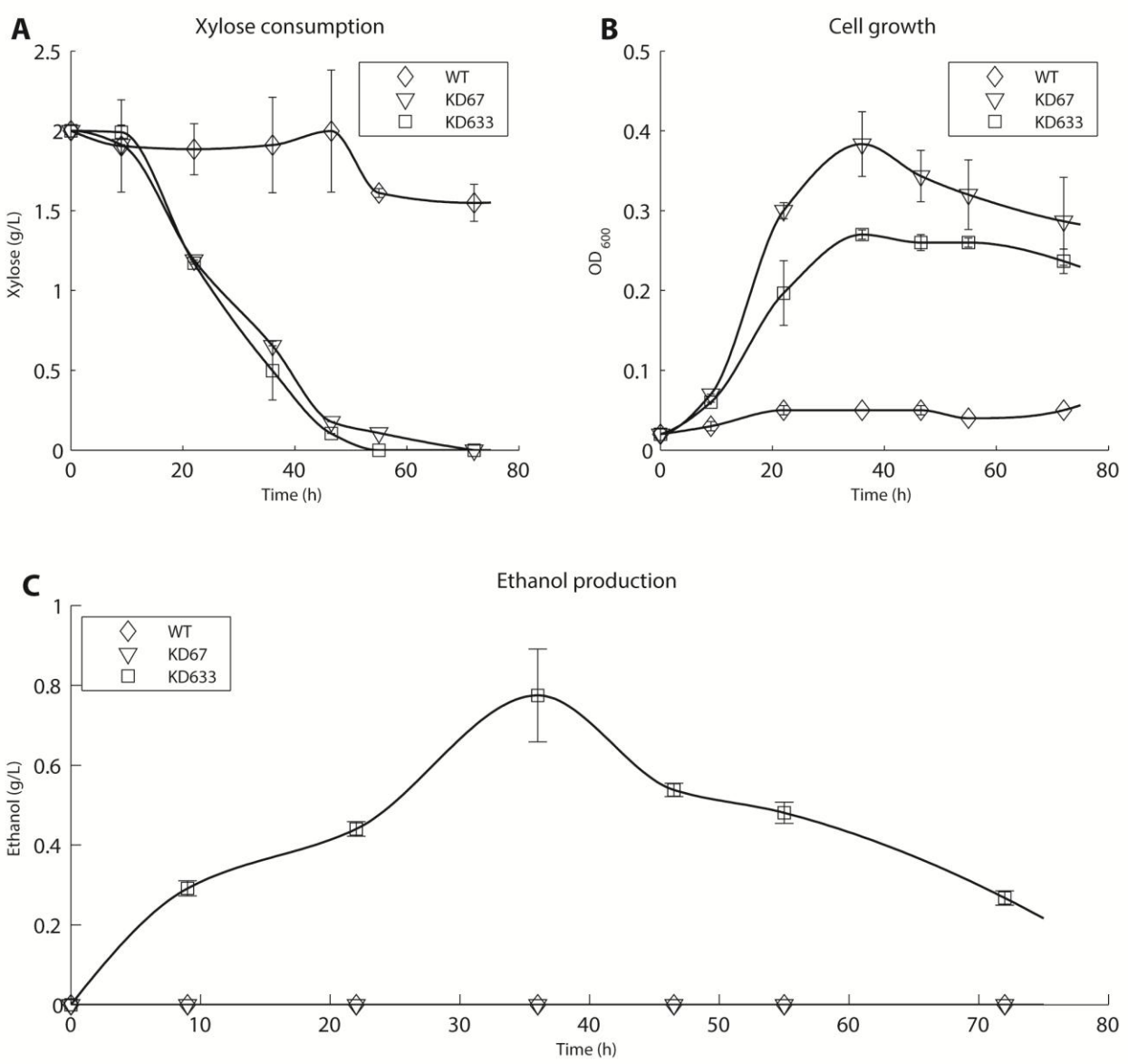

Figure 11: Strain analysis in rich medium containing approximately 0.1% xylose. Xylose concentration, growth, and ethanol concentration are shown for cultivation of wild-type (WT) *Z. mobilis* ZM4, recombinant *Z. mobilis* KD67 (xylose-digesting) and recombinant *Z. mobilis* KD633 (xylose-digesting, transporter-expressing). **A** Xylose concentration for each strain **B** Growth as determined by the OD_{600} **C** Ethanol concentration for each strain

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